

## Root Resorption in Streptozotocin-induced Diabetic Rats with Ligature-induced Periodontitis

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To determine the effect of diabetes on root resorption in periodontitis, we investigated odontoclast formation and root resorption in diabetic rats with periodontitis. Odontoclast formation was observed in three groups of F344 rats: Controls (C) were normal rats without diabetes or periodontitis; the periodontitis (P) group had mandibular first molars to be ligatured; the periodontitis with diabetes (PD) group was intravenously administered streptozotocin (50 mg/kg) to induce diabetes and had mandibular first molars to be ligatured. On days 3, 10, and 20 after ligature, tumor necrosis factor (TNF)- $\alpha$  and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) expression, odontoclast formation, and root

resorption areas were evaluated by immunohistochemistry, tartrate-resistant acid phosphatase staining, and hematoxylin and eosin staining, respectively. The PD group showed frequent urination, weight loss, and hyperglycemia. Numbers of TNF- $\alpha$ - and RANKL-positive cells were higher in the P and PD groups than in the C group. It was more prevalent in PD group on day 3. Odontoclast formation was greater in the P and PD groups than in the C group on days 3 and 10, then decreased to same level as the C group by day 20. Root resorption in the PD and P groups showed increases on days 3 and 10, respectively, compared to the C group. These results suggest that diabetes may transiently increase root resorption on day 3 with high expression of TNF- $\alpha$  and RANKL after periodontitis induction. This study could aid the understanding of root resorption in diabetic patients with periodontitis.

**Key words:** Diabetes Mellitus, Odontoclasts, Periodontitis, Root Resorption

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## Introduction

Tooth root resorption is a phenomenon occurring under physiological and pathological conditions, including tooth eruption, orthodontic tooth movement, and pulpitis [1,2]. In clinical reports of periodontitis, the resorption of root as well

as alveolar bone has been shown [3-5]. Raquel *et al.* [5] reported that the number and resorbed volume of teeth with resorbed roots were higher in severe periodontitis, suggesting that root resorption is associated with severity of periodontitis. During the process of root resorption, mononuclear and multinuclear tartrate-resistant acid phosphatase (TRAP)-positive odontoclasts participate in the destruction of dental hard tissues, such as cementum and dentin [6]. TRAP-positive multinucleated odontoclasts have been considered to form via the fusion of TRAP-positive mononuclear cells similar to osteoclasts [7]. Enzymatic properties of odontoclasts are similar to those of osteoclasts in terms of expression of H<sup>+</sup>-ATPase, cathepsin K, and matrix metalloproteinase-9 [2]. In addition, it has been reported that osteoclast differentiation factors can induce odontoclast formation, including receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and tumor necrosis factor (TNF)- $\alpha$  [2,8].

Many investigators have reported that the prevalence and severity of periodontitis is higher in the presence of diabetes, suggesting that diabetes aggravates periodontitis [9,10]. Higher levels of TNF- $\alpha$  and alveolar bone loss have been noted in rats with diabetes and periodontitis than with periodontitis alone [11]. In TNF inhibitor-treated rats with diabetes and periodontitis, it was documented that increased TNF- $\alpha$  expression was involved in more alveolar bone loss through dysregulated alveolar bone remodeling [12]. In our previous study, we also found dysregulated alveolar bone remodeling in diabetic rats with periodontitis [10,13]. Although effects of diabetes on alveolar bone in periodontitis have been suggested, the question of whether diabetes affects root resorption in periodontitis has not been addressed. Therefore, we investigated odontoclast formation and root resorption in streptozotocin-induced diabetic rats with ligature-induced periodontitis.

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## Materials and Methods

### Animals

Male 6-week-old F344 inbred rats (Oriental Bio, Gyeonggi-do, Korea) were acclimated for 1 week and then divided into three groups: control (C), periodontitis (P), and periodontitis with diabetes (PD) (6-8 animals per group). All animal procedure protocols were approved by the Institutional Animal Care and Use Committee of

Yonsei University (2014-0176-1).

### Induction of diabetes and periodontitis

To generate diabetes in the PD group, rats were intravenously administered streptozotocin (STZ, 50 mg/kg) dissolved in citrate buffer. One week after STZ injection, the P and PD groups were subjected to bilateral ligature of the mandibular first molars. To confirm diabetes induction, rats were fasted for 16 h and administered 2 g/kg glucose by oral administration. The Accu-Check active system (Roche Diagnostics, Mannheim, Germany) was used to measure blood glucose levels in accordance with the manufacturer's instructions. Body weight and food intake were measured during the experimental period.

### Hematoxylin and eosin (H&E) staining

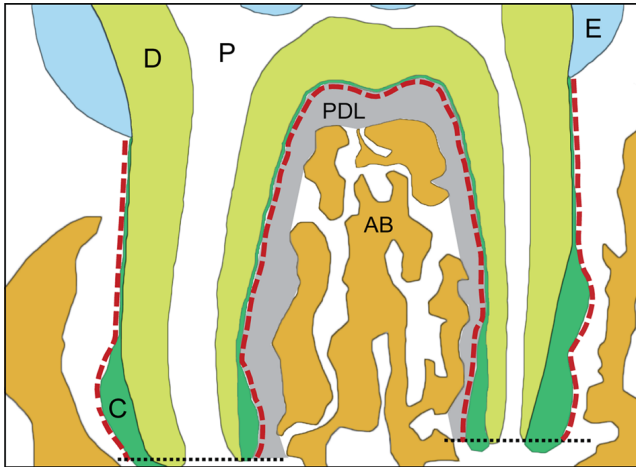
On days 3, 10, and 20 after periodontitis induction, the mandibles were collected and fixed in 10% neutral formalin. Mandibles were decalcified in 10% EDTA for 2 months and then embedded in paraffin. The sections that showed clear appearance of the dental pulp of the mesial and distal roots of the mandibular first molars were stained with H&E. To measure root resorption, resorption areas were measured along the mesial and distal root surfaces using Image-Pro software (Media Cybernetics, Silver Spring, MD, USA) under 200x magnification using light microscopy. Regions of interest (ROI) for measurement of root resorption are presented in Fig. 1 (red dotted line).

### Tartrate-resistant acid phosphatase staining

To determine odontoclast formation, TRAP staining was performed using commercial kits (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. According to Domon *et al.* [6], the number of mononuclear as well as multinuclear TRAP-positive odontoclasts was counted along the mesial and distal root surfaces under a light microscope (200x magnification) and are represented as odontoclast number per millimeter length of the root surface. ROI for the measurement of odontoclast formation are presented in Fig. 1 (red dotted line).

### Immunohistochemistry

Immunohistochemistry for TNF- $\alpha$  and RANKL was performed using the LSAB+ system-HRP kit (Dako, Carpinteria, CA, USA) as previously described [14]. The



**Fig. 1.** ROI in periodontal tissue of mandibular first molar. TRAP-positive cells and root resorption area were estimated along mesial and distal root surfaces (red dotted line). The TNF- $\alpha$ - and RANKL-positive cells were estimated in periodontal ligament area which was extended from apical furcation to apex of mesial and distal roots except alveolar bone (gray area). AB; alveolar bone, C; cementum, D; dentin, E; enamel, P; pulp, PDL; periodontal ligament.

sections were incubated overnight at 4°C with rabbit anti-TNF- $\alpha$  antibody (1:400 dilution; Abcam, Thousand Oaks, CA) and goat anti-RANKL antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The color was developed with substrate-chromogen, and methyl green was used as a counterstain. TNF- $\alpha$ - and RANKL-positive cells were counted in the first molar furcation under light microscopy (400x magnification). ROI in furcation extended from the apical furcation to the apex of mesial and distal roots (Fig. 1, gray area). The negative control omitted the primary antibody.

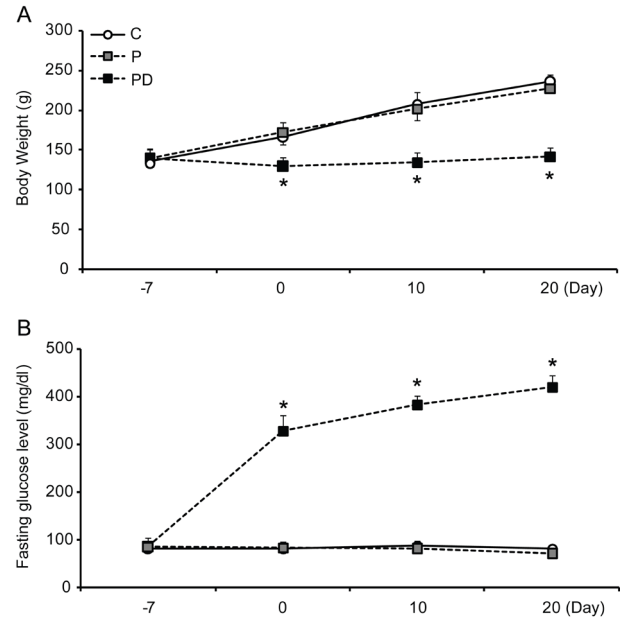
### Statistical analysis

Using nonparametric statistical analysis (Dunn's test, Kruskal-Wallis ANOVA), significant differences were examined among the three groups. All statistical analyses were performed using SPSS 12.0 (SPSS, Chicago, IL, USA), and a  $P$  value less than 0.05 was considered to be statistically significant. Data are expressed as the mean  $\pm$  standard error (SE).

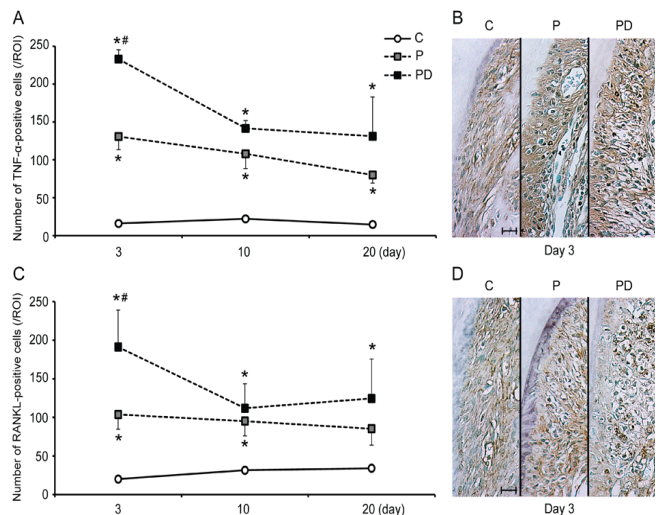
## Results

### Body weights and glucose levels

After STZ injection, the PD group showed frequent



**Fig. 2.** Diabetic characteristics of STZ-treated rats. Body weight changes (A). Rats were estimated body weight before (day -7) and after injection of STZ (days 0, 10, and 20). Fasting glucose levels (B). \*  $P < 0.05$  compared with the C group. C; control, P; periodontitis, PD; periodontitis with diabetes.



**Fig. 3.** TNF- $\alpha$  and RANKL expression. The number of TNF- $\alpha$ -positive cells in ROI (A). Representative images of TNF- $\alpha$  expression in ROI on day 3 (B). The number of RANKL-positive cells in ROI (C). Representative images of RANKL expression in ROI on day 3 (D). Bar = 10  $\mu$ m (400x magnification). \*  $P < 0.05$  compared with the C group. #  $P < 0.05$  compared with P group. C; control, P; periodontitis, PD; periodontitis with diabetes.

urination, weight loss, and hyperglycemia (over 300 mg/dl) under fasting conditions which are the typical symptom of diabetes during the experiment period (Fig. 2).

### TNF- $\alpha$ and RANKL expression

The number of TNF- $\alpha$ -positive cells increased in the P and PD groups compared with the C group during the experiment period (Fig. 3A, B). Three days after ligature, the number of TNF- $\alpha$ -positive cells preferentially increased in the PD group compared to the P group. But on days 10 and 20, there were no differences between the P and PD groups.

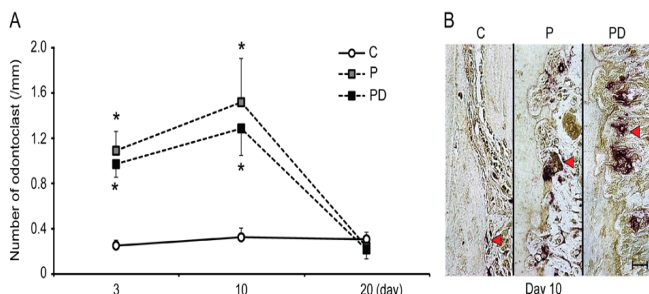
The number of RANKL-positive cells increased in the P and PD groups on days 3 and 10 compared with the C group (Fig. 3C, D). On day 3, the number of RANKL-positive cells was much higher in the PD group than in the P group. On day 20, there was an increase in the number of RANKL-positive cells in the PD group compared with the C group, but not in the P group.

### Odontoclast formation

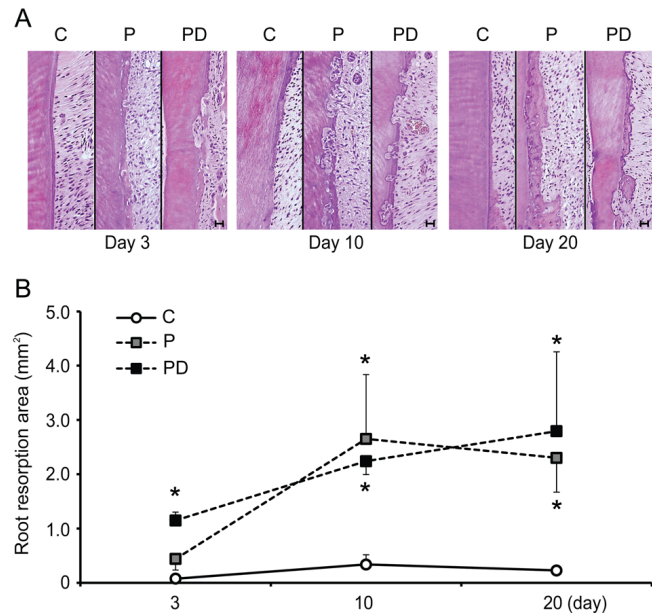
A few TRAP-positive odontoclasts were found in the C group (Fig. 4). The number of TRAP-positive odontoclasts was higher in the P and PD groups than in the C group on days 3 and 10, but was similar in the C group on day 20. There was no difference in the number of TRAP-positive odontoclasts between the P and PD groups throughout the experimental period.

### Root resorption

In the P and PD groups, the root resorption area was found in the cementum and dentin (Fig. 5A). While the PD group exhibited an increase in the resorption area during the experimental period compared with the C group, the P group showed an increase in the resorption area after day 10 as shown in Figure 5B. However, there



**Fig. 4.** Odontoclast formation. The number of TRAP-positive cells in ROI (A). Representative images of odontoclasts on day 10 (B). Red triangle indicate representative odontoclast in each group. Bar = 10  $\mu$ m (200x magnification). \*  $P < 0.05$  compared with the C group. C; control, P; periodontitis, PD; periodontitis with diabetes.



**Fig. 5.** Root resorption. Representative images of root resorption in ROI (A). Bar = 10  $\mu$ m (200x magnification). Root resorption area in ROI (B). \*  $P < 0.05$  compared with the C group. C; control, P; periodontitis, PD; periodontitis with diabetes.

were no differences in the resorption areas between the P and PD groups on days 10 and 20.

### Discussion

In this study, diabetes aggravated root resorption on day 3 only after periodontitis induction. This result suggested that diabetes did not consistently aggravate root resorption, but transiently increased root resorption. Diabetes also aggravated alveolar bone loss induced by periodontitis [11,13]. Aggravated alveolar bone loss by diabetes was consistently maintained from 2 to 6 weeks after periodontitis induction [11]. Taken together, these findings suggest that effects of diabetes on tooth roots may be weaker than on alveolar bone.

To determine the expression levels of odontoclast differentiation factors, we investigated the expression of TNF- $\alpha$  and RANKL in the first molar furcation of rats with periodontitis and diabetes. There was an increase in the number of TNF- $\alpha$ - and RANKL-positive cells in the P and PD groups compared to the C group. Higher expression of both cytokines in periodontitis was similar to results of previous studies [15,16]. Three days after

periodontitis induction, TNF- $\alpha$ - and RANKL-positive cells were more prevalent in the PD group than in the P group. These results are consistent with those of previous studies that showed higher expression levels of TNF- $\alpha$  and RANKL in gingival crevicular fluid of cases of periodontitis and diabetes than that of periodontitis alone [11,17]. The expression of TNF- $\alpha$  and RANKL increased in the gingival crevicular fluid of patients with severe root resorption after orthodontic treatment, suggesting that TNF- $\alpha$  and RANKL may play important roles in orthodontically induced inflammatory root resorption [18]. Also, an *in vitro* study showed that odontoclast formation by RANKL was more potent in the presence of TNF- $\alpha$ , indicating that TNF- $\alpha$  may aggravate root resorption during orthodontic treatment [8]. These findings imply that higher TNF- $\alpha$  and RANKL expression in the PD group could be related to increased root resorption.

To determine the relationship between increased TNF- $\alpha$  and RANKL expression and root resorption, we next estimated odontoclast formation and root resorption. On days 3 and 10, the number of odontoclasts in the P and PD groups was higher than in the C group, but there were no significant differences between the groups. Root resorption was found in 85% of patient's teeth with moderate periodontitis and in 93.55% of patient's teeth with severe periodontitis [5]. We also found increased root resorption in the P group on day 10 compared to the C group. The PD group also showed increased root resorption compared to the C group, but the time showing increased root resorption (day 3) was earlier than that of the P group. These results suggest that diabetes may transiently increase root resorption on day 3 with high expression of TNF- $\alpha$  and RANKL after periodontitis induction. RANKL increases resorbing activity as well as formation of osteoclasts and TNF- $\alpha$  can induce RANKL expression [19,20]. While the number of odontoclasts in the P and PD groups was similar, only the PD group showed root resorption on day 3 compared to the C group. This finding suggests a difference in the resorbing activity of odontoclasts between the P and PD groups. We assume that early increase of resorbing activity in odontoclast of diabetes might be related to the higher expression of RANKL and TNF- $\alpha$  on day 3. Dissimilar to our data, in an animal model of pulpitis, severe root resorption was observed on day 42 after pulp exposure in

diabetic rats compared to normal rats [21]. Therefore, to investigate changes in root resorption according to longer durations of diabetes and periodontitis, studies on the effects of diabetes over a longer experimental period are needed.

In this study, the PD group only showed increased root resorption on day 3 in the presence of high levels of TNF- $\alpha$  and RANKL, suggesting that diabetes may transiently aggravate root resorption by periodontitis. This study could aid in the understanding of root resorption in patients with diabetes and periodontitis.

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## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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